AD_____

Award Number: DAMD17-98-1-8644

TITLE: Identification of Novel Prostate Cancer-Causitive Gene Mutations by Representational Difference Analysis of Microdissected Prostate Cancer

PRINCIPAL INVESTIGATOR: Christopher Moskaluk, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22906

REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (15 Aug 98 – 14 Aug 99)

4. TITLE AND SUBTITLE
Identification of Novel Prostate Cancer-Causitive Gene Mutations by Representational Difference Analysis of Microdissected Prostate Cancer

5. FUNDING NUMBERS
DAMD17-98-1-8644

6. AUTHOR(S)
Christopher Moskaluk, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Virginia
Charlottesville, Virginia 22906

E-MAIL:
cam5p@Virginia.EDU

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Tissue from 23 radical prostatectomy operations has been procured. Microdissection of normal and cancer cells have been done for 2 specimens. Representational difference analysis has been completed on one sample yielding subtraction products which have been cloned and sequenced. Subsequent analysis showed that none of these products were from areas of homozygous deletion from the original tumor specimen. It was suspected that the published RDA protocol was not working properly. A control RDA with "spiked" target DNA sequences confirmed this. Using the same control DNA we have optimized the RDA protocol. The optimized protocol has been applied to two cases of primary prostate cancer, and the subtraction products are currently being analyzed. We have extended one of the stated goals of this project and will attempt to perform RDA on both RNA and DNA samples of primary prostate cancers. In this we can assay for transcriptional as well as genetic changes during prostate tumor progression. Towards this end, we have optimized an RNA extraction procedure from microdissected cells. We show that from as little as 2500 cells obtained from primary human tissue using laser capture microdissection we can isolate high molecular weight RNA suitable for this analysis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate Cancer	31
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Christopher D. Gmelek 9/8/99

PI - Signature Date

Table of Contents

Introduction

The subject of this research is the genetic basis of tumor progression in the human prostate. The purpose is to understand the molecular basis for the etiology of human prostate carcinoma, in the hopes of affecting changes in risk factors that affect this disease, in refining diagnostic tests that can help guide therapy and providing information useful in the rational design of therapeutic agents. The scope of this work entails the procurement of high quality tissue samples of human prostate cancer, microdissecting pure sample of prostate cancer and normal cells from this tissue, the efficient extraction of nucleic acids from these microsamples, and the subsequent performance of the technique known as representational difference analysis (RDA). RDA is capable of determining the differences between two complex but highly related genomes (such as cancerous and benign prostate epithelial cells). These differences are likely to contain the key molecular constituents that are responsible for the malignant behavior of prostate cancer cells. Most of our efforts are focused on identifying areas of homozygous deletion in prostate cancer genomes, which are likely to harbor tumor suppressor genes involved in the pathogenesis of this disease.

Body of Summary

Tissue procurement

In addition to the bank of tissue we had on hand prior to the start of this project, we have continued to collect tissue samples from radical prostatectomy specimens with an enhanced protocol to ensure the best quality tissue in terms of RNA and DNA preservation. We have a tissue procurement technician now scrubbed and waiting in the operating room to receive the prostate at the first opportunity. The specimen is taken immediately to the Surgical Pathology suite where the principle investigator (C.M.) performs the necessary gross examination and sectioning of margins to comply with IRG regulations for appropriate medical care, then tissue samples are immediately obtained, frozen in liquid nitrogen, and where necessary, are examined by cryostat sectioning and histologic staining for the presence of appropriate cancerous and benign tissue elements. We have procured an additional 23 specimens in this manner since the start of the funded project.

Representational difference analysis of a primary human prostate cancer

Our first RDA was performed on microdissected specimen of a metastatic Gleason grade 5 cancer. The initial amplicons of both tumor and normal appeared to be adequate as evidenced by PCR products of appropriate size, and the presence of distinct bands that represent repeated elements, which were identical between the two specimens. Three rounds of RDA were performed, The results of round three was a marked reduction in the amplicon population, with the remaining DNA products in a tight cluster between 300-400 bp in size. However, it was not clear that this population represented discrete subtraction products. This DNA population was subcloned into a plasmid vector. 50 clones were replica plated, lysed onto filter supports, and then blotted in parallel with probes made from driver (tumor) and tester (benign) amplicons. None of the subclones showed a differential hybridization pattern. Another 24 clones were examined by DNA sequencing. We obtained 8 unique sequences. From these sequences, synthetic oligonucleotides were designed as polymerase chain reaction (PCR) primers. Subsequent PCR assays were performed on the original normal and tumor DNA samples. None of the subtraction products were found to represent homozygous deletions.

These results could be accounted for if our initial specimen had no homozygous deletions, or if the RDA procedure was sub-optimal. Since we did not have a test prostate cancer with a known homozygous deletion AND matched DNA from non-neoplastic tissue, we decided to test our RDA protocol on a sample of genomic DNA which had been "spiked" with a small amount of exogenous foreign DNA, to serve as the target DNA.

Optimization of the RDA protocol

We spiked a 5 ug sample of human DNA with 50 pg of bacteriophage lambda DNA. This represents a $6 \times 10^9 : 1$ molar ratio of driver DNA to target DNA, and is a rigorous test of the efficiency of any differential subtraction technique. We applied three rounds of RDA to the spiked sample, using it as the tester DNA population, with a "non-spiked" DNA sample serving as the driver population. We used Bgl II as the digesting restriction enzyme to form the amplicon population. This digest yields a 700 bp product from the bacteriophage lambda DNA that should appear in the RDA subtraction products. The initial published protocol of RDA had used this

test, and found that this DNA fragment should be seen as a distinct band in the PCR difference products after 2 rounds of RDA.

Our initial test showed that the published conditions in our hands failed to deliver the expected results. Even after three rounds of RDA, the expected band did not materialize in the subtraction products. We began a systematic overhaul of the multi-step RDA protocol to determine what steps were failing to perform to specifications. Our analysis indicated that determination of amplicon DNA concentration by spectroscopy did not appear to be as accurate as gel electrophoresis against known standards, that twice as much DNA and twice the time specified in the original protocol was required for reproducible and efficient hybridization of the amplicon populations, and that titration PCR experiments were required after each hybridization to optimize the production of post-hybridization PCR amplification of subtraction products. The results of our trouble shooting is reflected in the optimized RDA protocol contained in the Appendix. Using the optimized protocol, we have observed the appearance of the lambda DNA as the major specific difference products after two rounds of RDA (from an initial molar ratio of greater than 10^6), as confirmed by Southern blots using bacteriophage DNA as the probe.

RDA subsequent to optimization

We have just recently completed the optimization of the RDA protocol. We are currently applying the RDA protocol to two freshly microdissected samples of prostate cancer: the original metastatic Gleason grade 5 cancer and a Gleason grade 3 cancer confined to the prostate. After two rounds, the optimized RDA protocol is yielding distinct bands of a greater size distribution than our initial protocol. We are currently in the process of subcloning and analyzing these subtraction products as possible markers of homozygous deletion.

Optimization of RNA extraction from microdissected prostate cancer

The RDA protocol has the ability to determine the differences in transcription profiles as well as differences in genomic content between two populations of cells. As an extension of our genetic analysis of prostate cancer, we intend to extract RNA from microdissected samples of prostate cancer and benign prostate epithelial cells, and perform RDA on the subsequent cDNA libraries of each cell population. Researchers involved in the Cancer Genome Anatomy Project at the National Cancer Institute have recently shown that complex RNA samples can be successfully obtained from microdissected samples of human prostate tissue. We have adopted and optimized their protocol in our laboratory in anticipation of RDA analysis. Our protocol can be found in the Appendix.

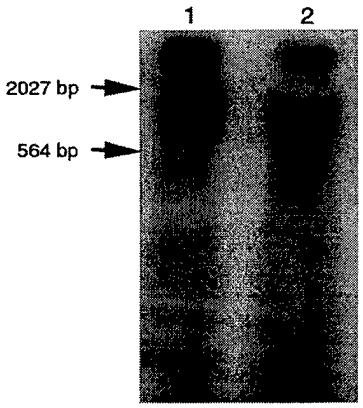

Figure 1: Autoradiogram of ^{32}P labeled cDNA obtained from 2500 microdissected cells. Lane 1: RNA sample not treated with DNases prior to cDNA synthesis. Lane 2: RNA sample treated with DNase I and Exonuclease III prior to cDNA synthesis.

Figure 1 shows gel electrophoresis of radioactively labeled cDNA obtained from laser capture microdissected tissue samples of 2500 cells. The majority of cDNA is in the 600-2000 bp size range, suggesting that significant degradation had not occurred prior to, and during, RNA extraction. This autoradiogram came from an optimization experiment in which we determined that the addition of a DNase step was crucial in obtaining RNA samples from contaminating genomic DNA. This can be seen in Figure 2, where coupled reverse transcriptase-PCR assays for β-actin showed that only in samples treated with DNase was there reverse transcriptase-specific PCR amplification. We are now ready to apply these procedures to matched microdissected samples of benign and normal prostate epithelial cells in preparation for RDA analysis.

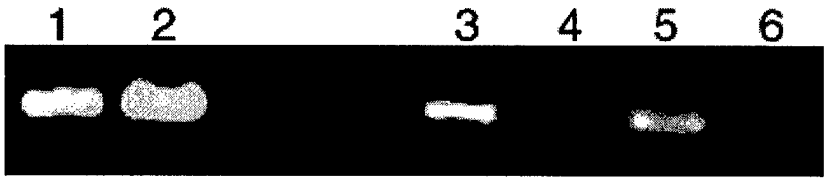

Figure 2: RT-PCR assays of microdissected tissue samples. Lanes 1& 2 correspond to the RNA sample in Lane 1 of Figure 1, which was not treated with DNases. A PCR product appears in RNA with (Lane 1) and without (Lane 2) reverse transcriptase treatment, signaling the presence of contaminating genomic DNA. Lanes 3 & 4 correspond to the RNA sample in Lane 2 of Figure 1, which was treated with DNases. A PCR product appears only with RNA treated with reverse transcriptase (Lane 3), and not in the RNA sample not reverse transcribed (Lane 4), consistent with no significant contamination with genomic DNA. Lane 5 is a positive PCR control, with 0.15 ng input genomic DNA. Lane 6 is a negative PCR control, with no template added. The PCR product corresponds to 587 bp of β-actin sequence. Each PCR reaction contains 375 cell equivalents of cDNA.

DAMD17-98-1-8644
Principal Investigator: Christopher Moskaluk
Annual Summary
Report date: September 1999

Appendices

Key Research Accomplishments
Reportable outcomes
Optimized protocol for representational difference analysis
Protocol for RDA amplicon generation from microdissected tissue
Protocol for RNA isolation from microdissected tissue

Key Research Accomplishments

- Optimization of protocol for Representational Difference Analysis (RDA)
- Application of RDA to two sets of microdissected prostate cancer samples
- Optimization of protocol for RNA isolation from microdissected tissue specimens

Reportable outcomes

Procurement of tissue from 23 radical prostatectomy specimens

General notes

Although PCR reactions are set up in the PCR hood, the amplicons and Taq polymerase are added to the reactions at your benchtop or at the PCR machine using your personal pipettors. Taq polymerase working dilutions are made fresh from concentrated stock for each use. AMPLICONS ARE NEVER, NEVER BROUGHT INTO THE PCR ROOM. You must exercise precise pipeting technique to avoid contamination of the pipet tips and avoid aerosols that can contaminate other samples. Don't touch your pipet tips to the sides of tubes. Use fresh tips between samples. Release the tension on the pipettors inside the tube or over the trash can, NEVER over the tubes or over the working area. Wipe the ends of your pipet tips with a 20% bleach solution after pipeting amplicons. Aerosol barrier (filter) pipet tips should be used in the following instances: 1) when pipeting relatively large volumes (150 ul) of amplicons, as in during phenol extractions and 2) when vigorously resuspending DNA pellets. Sections in the protocol where these tips are to be used are designated by **FPT** (filter pipet tip). At other times, when pipeting small volumes of amplicons, filter tip pipet tips are not necessary.

Oligonucleotides: RBgl24, NBgl24 & JBgl 24 oligonucleotides are used as both ligation adapters and PCR primers in this protocol. We use unpurified oligos as PCR primers and use reverse phase purified oligos as ligation adapters. When following this protocol, use unpurified oligonucleotides unless purified oligos are specified.

Organic extractions: Phenol (equilibrated against a Tris buffer w/ pH $\geq$ 8.0) is added in a volume equal to the DNA solution. The tube is capped, vortexed for 15 seconds and spun in a microcentrifuge at top speed for 4 minutes. The top aqueous layer is transferred to a fresh tube, avoiding any milky interface.

Alcohol precipitation: Whenever "alcohol precipitation" is indicated in the protocol, ALL of the following steps (including washes) are meant to be performed. The DNA, salt solution, and any carrier to be used are combined, the tube is closed, vortexed, then spun briefly to settle the contents. The tube is opened and then the alcohol is added. The tube is inverted and vortexed several times to thoroughly mix the contents. Incubate in the -80° C freezer for at least 15 min., then spin for 10 min. at top speed in a microcentrifuge. The supnt. is removed and 800 ul of 70% EtOH is added. The tube is spun briefly in the microcentrifuge (< 1 min.), the supnt. removed, and the wash repeated one more time. After the last step, all liquid is removed with a pipettor. Pellets may be resuspended in a damp state if all visible fluid is removed. Pellets may be dried BRIEFLY by placing them in the 45° C drying oven. Over-drying may result in pellets that are difficult to resuspend.

Gel electrophoresis: Unless otherwise specified, use 2% agarose gels. It is best to use an EC Minicell (30 ml), with 2 to 3 rows of wells. You can reuse the gels several times, but be sure there is extra ethidium in the lower running buffer to replace the ethidium in the gel. **For gel quantitation:** 1) use a freshly prepared gel using 8 well combs, 2) Run blue dye at least 1 cm, but no more than 2 cm, from the wells, and 3) after electrophoresis soak the gel in 2 ug/ml EtBr in 1X TAE for 10-15 minutes (do not wait too long or the DNA will diffuse). The gel is visualized under UV light in the AlphaImager 2000, and

the image is examined using digital integration in the "expose" capture mode with the "show saturation" option selected. Choose the exposure time immediately before saturation is detected. Turn off the saturation option, then capture the image using the "freeze" function. Using the "spot denso " function in Toolbox 3#, enclose the test samples as well as the molecular weight standards with boxes of the same size (use the Copy function). Generate a standard curve using the molecular weight marker lanes; if the standard curve is not roughly linear, consider repeating the gel electrophoresis. Use the data from test lanes ONLY if the test samples fall WITHIN the boundaries of the standard curve.

Determining number of amplification cycles: The plateau phase of PCR occurs when the amount of primer, dNTPs and/or Taq polymerase becomes limiting, and single strand DNA is no longer primed or replicated to completion. This manifests itself as a decrease in the rate of product accumulation and sometimes even as a decrease in PCR product. In some instances, the partially completed PCR products will form slower migrating complexes that manifest as "smearing towards the well" in gel electrophoresis. Therefore, if the same or similar amount of PCR product is observed in 2 sequential samples, it is better to choose the lower # of cycles.

Abbreviations:

NEB	New England BioLabs
BM	Boehringer Mannheim
NaOAc	Sodium Acetate
FPT	filter pipette tip

Genomic DNA Preparation

Digest 5 ug (A_{260} reading) of genomic DNA with 20 units of Bgl II enzyme in a volume of 200 ul, using the restriction buffer supplied by the manufacturer, for 2 to 4 hours at 37°C.

Phenol extract (**FPT**).

Alcohol ppt. as described above, using 2 ul of 5 mg/ml tRNA, 20 ul 3 M NaOAc, 400 ul EtOH

Resuspend in 90 ul loTE (**FPT**). Add 10 ul of 10X restriction buffer and 20 units Bgl II. This second step ensures the complete cleavage of genomic DNA.

Incubate 2-4 hrs. at 37° C.

Add 100 ul loTE.

Phenol extract and alcohol ppt. as above.

Resuspend pellet in 17 ul loTE.

Load 2 ul on a 1% agarose gel, with 0.1. 0.3 and 0.6 ug of genomic DNA standards. Include a lane with 1 ug of λ Hind III MW ladder and a lane with 2 ul of 0.1 mg/ml tRNA. (Genomic DNA standards consist of human genomic DNA cut w/ a restriction enzyme (Bgl II), phenol extracted, alcohol precipitated without carrier, and the concentration determined by spectroscopy at 260 nm).

Determine concentration with the spot densitometry function on the AlphaImager 2000. Be sure not to include the tRNA band at the bottom of the lanes in your quantitation.

Place 5 ul of 100 uM RBgl12 oligonucleotide (**purified**), 5 ul of 100 uM RBgl24 oligonucleotide (**purified**) in a 1.5 ml microfuge tube.
Add 1 ug of digested genomic DNA, 3.5 ul of 10X ligation buffer (NEB) and enough loTE to bring volume to 34 ul.
Place mineral oil in dry heating block and bring temp. to 55° C.
Place tube in heat block, then remove block from heater and place in refrigerator.
Let temperature fall to 12° C.
Spin tube briefly.
Add 1ul 400u/ul T4 DNA ligase (NEB)
Incubate at 15° C for at least 8 hours.
Heat inactivate at 65° C for 10 minutes. Spin down.
Dilute to 1 ml using loTE.

Titration of amplicon conditions

Set up 6 PCR reactions (3 reactions at 2 different extension temperatures):

10X RDA	14.5 ul	→	101.5
10 mM dNTPs	6 ul		42
50 mM $MgCl_2$	12 ul		84
5 M Betaine	15 ul		105
100 uM RBgl24	1.5 ul		10.5
loTE	81 ul		567

130 ul premix per tube.
Add 15 ul of diluted genomic DNA preparations (neg control; control DNA; target DNA), or loTE (negative controls) to appropriate tubes.
Add 2 drops oil. Place in PCR machine and use programs "AMP72" and "AMP69". The machine is set to SIM TUBE; enter a calibration factor of 200.
72° C X 3 min. (this dissociates the RBgl12 oligonucleotide), hold at 72° C.
Add 5 ul of 5 u/ul Taq in 1X RDA buffer (fresh dilution). Mix by pipeting.
72° C X 5 min (this fills in ends of DNA fragments, copying the RBgl24 sequence, to allow subsequent PCR to occur).
Cycles: 94° C X 1 min, 72° C X 3 min or 94° C X 1 min, 69° C X 3 min
16 cycles. Hold. Remove 15 ul
2 more cycles (18). Hold. Remove 15 ul.
2 more cycles (20). Hold. Remove 15 ul.
2 more cycles (22).
Load 5 ul of each aliquot onto a 2% agarose gel. Include a lane with 1 ug λ HindIII markers.
Pick the number of cycles which first gives maximal amplification products in the 500 to 2000 bp range just prior to or just at the beginning of the plateau phase of PCR.

If maximal PCR amplification has not occurred, set up a new titration reaction to assay 22, 24 and 26 cycles.

Amplicon Generation

Set up 24 reactions as above for driver amplicons.
Set up 8 reactions as above for tester amplicons.
Perform PCR as above (72° C pre-incubation, etc.) except use one less cycle than the optimal number determined.
After the cycles are over, prepare the following premix for each tube:

		For 35 reactions
100 uM RBgl24	0.75 ul →	26.25
10X RDA	1 ul	35
10 mM dNTP	2 ul	70
loTE	4.25 ul	148.75
5 u/ul Taq polymerase	2 ul	70

Preheat reaction tubes to 78° C.
Add 10 ul of premix to each tube. Mix thoroughly with second pipettor.
Run 1 cycle: 94° C X 1 min, 72° C X 10 minutes.
Combine two 160 ul PCR reactions into one 1.5 ml microfuge tube (**FPT**).
Phenol extract (**FPT**).
Alcohol ppt.: 30 ul 3 M NaOAc, 600 ul EtOH.
Resuspend each pellet in 50 ul loTE (**FPT**). Combine resuspended amplicons.
Load 1 & 2 ul of amplicons on 2% gel. Include lanes with 1 ug λ HindIII markers, and 0.2, 0.5, and 0.8 ug of LMW markers.
Estimate concentration after running blue dye 1-2 cm from the well by digital image quantitation.

Tester amplicon preparation

Digest 10 ug of amplicons in 200 ul final volume using 100 units Bgl II. Mix final reaction thoroughly with gentle pipeting (**FPT**).
Incubate 37° C X 5 hours.
Phenol extract (**FPT**).
Alcohol precipitate: 2 ul 5 mg/ml tRNA, 20 ul 3 M NaOAc, 400 ul EtOH.
Resuspend in 20 ul loTE.
Add 7 ul 5X PCR-clean glycerol loading buffer and mix. Load into a 50 ml 1.5% low melting point agarose (NuSieve Agarose works best) gel (10 well comb) made in the Bio 101 gel apparatus designated for RDA. The apparatus should be filled with fresh 1X TEA buffer containing 0.5 mg/ml EtBr. Load 2 ug of λ Hind III markers (10 ul 200ng/ul) and 0.5 ug LMW markers (5 ul 0.1 ug/ul) in adjacent wells. In another well, load 5 ul of 0.1 mg/ml tRNA. Place gel unit in ice bath, and perform electrophoresis at 50V. Stop electrophoresis when blue dye has migrated 2 cm from wells.

Take gel to the UV transilluminator in Room 3922 OMS. Do NOT use the transilluminator in the photodocumentation system in our lab. Protect your eyes and face with the UV face shield, and cover all exposed skin on your hands and arms. IT IS IMPORTANT TO MINIMZE THE EXPOSURE OF THE DNA TO UV LIGHT!! Work as quickly as possible, turn the UV light off whenever you are not visualizing the DNA. Excise amplicons in the 100-2000 bp range using a clean razor blade. Exclude as much of the tRNA band as possible. Trim away all excess agarose that does not contain DNA.

Purify DNA using agarase/Centricon-100 method (see separate protocol).

You should retrieve <100 ul. Measure volume with a pipetman.

Load 10% of purified amplicons on same gel as driver amplicons (see below) and obtain DNA concentration. If concentration is too low, alcohol precipitate.

Set up ligation:

1ug of gel purified amplicons
5 ul 100 uM JBgl24 (**purified**)
5 ul 100 uM JBgl12 (**purified**)
3.5 ul 10X ligation buffer (NEB)
lo TE to 34 ul final volume

Hybridize primers to DNA using 55° C hot block as described in "Genomic DNA Preparation" section.

Spin tubes down after 12° C, add 1 ul 400 u/ul T4 DNA ligase (NEB), and incubate at 15° C 8 hours to overnight.

Heat inactivate at 65° C for 10 minutes. Spin down.

Add 65 ul loTE (final volume 100 ul).

Add 1 ul to 9 ul loTE to make 1:10 dilution

Test PCR:

10X RDA	4.5 ul	→	20.25
10mM dNTPs	2 ul		9
50 mM $MgCl_2$	4 ul		18
5 M Betaine	5 ul		22.5
loTE	32.5 ul		146.25

In 2 PCR tubes, place 0.5 ul 100 uM RBgl24 +0.5 uL loTE, in another 2 tubes place 0.5 ul 100 uM JBgl24+0.5 uL loTE.

Add 43 ul of premix to each tube.

Add 1 ul of the 1:10 dilution of JBgl amplicon ligation reaction to the "test" tubes, and 1 ul loTE to the negative control tubes.

2 drops oil. Spin.

Place in PCR machine and use "TEST70" program in SIM TUBE mode with a calibration factor of 100.

72° C X 3 min. Hold at 72° C.

Add 5 ul 2 u/ul Taq in 1X RDA.

72° C X 5 min.
15 cycles: 94° C X 1 min, 70° C X 3 min
Load 10 ul of each reaction on a 2% gel.
There should be an equal or greater amount of amplicons in the JBgl reaction than in the RBgl reaction. If not, redo tester amplicon preparation.

Driver amplicon preparation

Digest 200 ug of amplicons in 800 ul final volume using 800 units Bgl II (5 u per ug DNA). Mix final reaction thoroughly with gentle pipeting (**FPT**).
Incubate 37° C X 5 hours.
Split reaction into 2 tubes (400 ul each)
Phenol extract (**FPT**).
Purify amplicons by centrifugation in a Centricon 100 unit. Place aqeous phase in sample reservoir (upper chamber). Add loTE to raise volume to 2 ml mark on reservoir. Centrifuge at 700 x g for 20 minutes (IEC rotor 809, 2500 rpm). Remove filtrate from filtrate vial (lower chamber). Add fresh loTE to upper chamber to raise volume to 2 ml mark. Centrifuge as before. There should only be a scant amount of fluid covering the membrane at this point. If there is excess liquid, spin at 700 x g for a few more minutes. Remove lower chamber, then invert upper chamber onto a clean recovery tube. Centrifuge 700 x g for 4 minutes (2500 rpm). You should recover <100 ul.
Add 2 ul of purified amplicons to 18 ul loTE to make 1:10 dilution.
Load 2, 4 and 6 ul of 1:10 dilution on 2% agarose gel. Include a lane with 0.5 ug undigested amplicons. Include 0.2, 0.5 and 0.8 ug LMW markers.
Determine concentration after running blue dye 1-2 cm from well by digital image quantitation.
Run blue dye 4 cm from the well, and verify that repeat bands present in the amplicons are slightly smaller in the digested lanes as compared to the undigested lane.

Hybridization #1

In a clean 1.5 ml microcentrifuge tube, combine:
80 ug Bgl II digested driver amplicons
1 ug JBgl-ligated tester amplicons (100 ul)
loTE to bring volume to 200 ul.

Phenol extract (**FPT**).
Alcohol precipitate: 20 ul 3 M NaOAc, 400 ul EtOH (NO CARRIER).
After washing, be sure to dry pellet thoroughly.
Under direct observation, resuspend pellet in 3.5 ul 3X EE buffer by pipeting gently up and down. It should take several minutes to accomplish this. Avoid leaving droplets in pipet tip. The solution should be slightly viscous, and hold a tight meniscus. It may be cloudy, but should have no particulates. Final volume should be close to 4 ul.
Transfer to 0.2 ml PCR tube. Overlay with 1 drop oil.
Set one PCR block to 98° C, another to 67° C.
Preheat a small aliquot of 5 M NaCl at 67° C.

Incubate resuspended DNA at 98° C X 7 minutes.
Perform the following steps as quickly as possible:
Using Wheaton 0.2-2 ul "Socorex" micropipetor, preheat pipet tip in 5 M NaCl by pipeting up and down a few times.
Remove a 1ul aliquot of 5 M NaCl.
Remove DNA tube from 98° C, add the 1 ul of NaCl, pipet once or twice to mix, and place in 67° C block.
Incubate 40 hours at 67° C.

Hyb1-PCR1 (H1P1)

Carefully remove as much oil as possible from the hybridization reaction.
Add 8 ul 5 mg/ml tRNA to Hyb1. Mix by pipetting up and down gently. Avoid getting droplets caught in pipet tip.
Add 10 ul loTE. Under direct observation, mix by pipeting gently until refraction lines are no longer seen.
Transfer to clean 1.5 ml microcentrifuge tube.
Wash hybridization tube with 80 ul loTE, and add to previous solution.
Add 300 ul loTE. Mix by pipeting gently (**FPT**). (400ul final volume)

100 uM JBgl 24	1.5 ul.....3.75
10X RDA	14.5 ul.....36.25
10mM dNTPs	6 ul.....15.0
50 mM $MgCl_2$	12 ul.....30.0
5 M Betaine	15 ul.....37.5
loTE	46 ul.....115

95 ul premix into each of two 0.2 ml PCR tubes.
50 ul diluted Hyb1 into each tube.
2 drops oil. Spin down.
Place in PCR machine and use "H1P1" program in SIM TUBE mode with a calibration factor of 200.
85° C X 2 minutes (this step melts heteroduplexes that have hybridized to internal repetative elements).
Hold at 72° C.
Add 5 ul 5 u/ul Taq. Mix thoroughly with second pipettor.
72° C X 5 minutes (this step fills in the 3' end of hybridized DNA fragments)
10 cycles: 94° C X 1 min, 70° C X 3 min.
Final incubation at 72° C X 5 min.

Mung Bean Nuclease Treatment of H1P1

Combine the two tubes of H1P1.
Phenol extract (**FPT**).

Alcohol precipitate (30 ul 3M NaOAc, 600 ul EtOH).
Resuspend pellet in 5 ul loTE (**FPT**).

Add:
4 ul 10X MBN buffer (NEB)
29 ul loTE
2 ul Mung Bean Nuclease (10 u/ul, NEB)

Mix gently with pipette.
Incubate 30 min at 30° C.
Spin down, then add 160 ul 50 mM Tris pH 8.8.
Phenol extract and alcohol precipitate.
Determine concentration by running an 2% agarose gel with 0.2, 0.4, 0.8 ug LMW markers and with 2 ul, 5 ul, 10 ul 1:5 H1P2 amplicon dilution. Determine volume required for 50ng.

Hyb1-PCR2 (H1P2)

3 different amounts of digested amplicons will be tested in PCR reactions::

10X RDA	14.5 ul.....50.75
10mM dNTPs	6 ul.....21
50 mM $MgCl_2$	12 ul.....42
5 M Betaine	15 ul.....52.5
loTE	66 ul.....231
100 uM JBgl24	1.5 ul.....5.25

Into 3 PCR tubes, add 115 uL premix and then add:
30 ul of digested amplicons to one tube
15 ul digested amplicons + 15 ul loTE to the next tube
to the last tube add 50 ng amplicons + loTE to bring final volume to 145 ul.
Add 2 drops oil, spin down.
Place in PCR machine and use "H1P2TST" program in SIM TUBE mode with a calibration factor of 200.
85° C X 3 minutes. Hold at 78° .
Add 5 ul 5 u/ul Taq in 1X RDA buffer.
Cycles: 94° C X 1 min, 70° C X 3 min.
16 cycles. Hold. Remove 15 ul.
3 more cycles (19). Hold. Remove 15 ul.
3 more cycles (22). Hold. Remove 15 ul.
3 more cycles (25). Hold. Remove 15 ul.
3 more cycles (28). Hold. Remove 15 ul.
3 more cycles (31).

Load 5 ul of aliquots on 2% gel. Choose the optimal number of cycles and DNA concentration to use.

Preparation of Round 1 Subtraction Products

10X RDA	36.25 ul
10 mM dNTPs	15 ul
50 mM Mg Cl_2	30 ul
5 M Betaine	37.5 ul
100 uM JBgl24	3.75 ul
loTE	165 ul

115 ul premix each into 2 PCR tubes.
Add optimal diluted MBN-treated H1P1 + loTE to bring final volume to 145 ul.
2 drops oil
85° C X 3 min. Hold 78° C
Add 5 ul 5 u/ul Taq polymerase.
Use one less than the optimal number of cycles determined above.
After the cycles are over, prepare the following premix:

100 uM JBgl24	1.5 ul	→	3.75
10X RDA	1 ul		2.5
10 mM dNTP	2 ul		5
loTE	3.5 ul		8.75
5 u/ul Taq polymerase	2 ul		5

Preheat reaction tubes to 78° C.
Add 10 ul of premix to tubes.
Run 1 cycle: 94° C X 1 min, 70° C X 3 minutes, 72° C X 10 minutes.

Combine tubes.
Phenol extract (**FPT**).
Alcohol precipitate: 30 ul NaOAc, 600 ul EtOH. Do not add carrier.
Resuspend in 50 ul loTE (**FPT**). If the solution is cloudy, centrifuge 3 minutes and transfer the supernatant to a fresh tube.
Make a 1:10 dilution (2 ul into 18 ul loTE). Load 2,5 and 10 ul of 1:5 dilution of H1P2 with 5 ul of 5X loading buffer in a **3%** agarose gel with 0.2, 0.5 and 0.8 ug of LMW markers. Also load 1 ug of tester amplicons in adjacent lane. Determine DNA concentration of H1P2 stock. After gel electrophoresis, the repeat bands present in the tester amplicons should not be present in the H1P2 amplicons, but new additional distinct bands should be visible. If not, recheck amplicon concentrations and redo hybridization.

Digest 10 ug of H1P2 in 200 ul with 100 units Bgl II X 5 hours at 37°C, using buffer supplied by the manufacturer.
Phenol extract (**FPT**).

Concentrate amplicons on Centricon-100 device, and determine DNA concentration, as described in "Driver Amplicon Preparation".

Make two dilutions of stock digested amplicons: 20 ng/ul and 100 ng/ul.
Set up two ligation reactions (one w/ 20 ng/ul dilution, one with 100 ng/ul dilution):
5 ul of dilution of Bgl II-digested H1P2 amplicons
5.25 ul 100 uM NBgl24 (**purified**)
5.25 ul 100 uM NBgl 12 (**purified**)
10.5 uL loTE
3 ul 10X ligation buffer (NEB)

Hybridize primers to DNA using 55° C hot block as described in "Genomic DNA Preparation" section.
Spin tubes down after achieving 12° C, add 1 ul 400 u/ul T4 DNA ligase (NEB) to each tube, and incubate at 15° C 8 hours to overnight.
Heat inactivate at 65° C for 10 minutes. Spin down.
Add 50 ul 20 ug/ml tRNA in loTE (final volume 80 ul) to each tube. Make an additional 1:5 dilution of the 100ng/ul ligation reaction (2 ul into 8 ul loTE).

Test PCR:

10X RDA	4.5 ul	→	31.5
10mM dNTPs	2 ul		14
50 mM $MgCl_2$	4 ul		28
5 M Betaine	5 ul		35
loTE	27.5 ul		192.5

In 3 PCR tubes, place 0.5 ul 100 uM JBgl24 + 0.5 uL loTE, in another 3 tubes place 0.5 ul 100 uM NBgl24 + 0.5 uL loTE.
Add 43 ul of premix to each tube.
Add 1 ul of the 20 ng/ul ligation each to a tube with JBgl24 primer and to a tube with NBgl24 primer.
Add 1 ul of the 1:5 dilution of the 100 ng/ul ligation each to a tube with JBgl24 primer and to a tube with NBgl24 primer.
Add 1 ul loTE to the remaining tubes (negative controls).
Add 2 drops oil to each tube. Spin.
Place in PCR machine and use "TEST72" program in SIM TUBE mode with a calibration factor of 100.
72° C X 3 min.
Add 5 ul 2 u/ul Taq in 1X RDA buffer.
72° C X 5 min.
15 cycles: 94° C X 1 min, 72° C X 3 min
Load 10 ul of each reaction on a 2% gel.
There should be an equal or greater amount of amplicons in the NBgl reactions than in the JBgl reactions. If not, redo tester amplicon preparation. Determine which ligation reaction yielded the best differential between N and J amplification, and/or the best yield of amplicons.

Hybridization #2

In a clean 1.5 ml microcentrifuge tube, combine:
80 ug Bgl II digested driver amplicons
100 ng of NBgl ligated round 1 subtraction amplicons (80ul)
loTE to bring volume to 200 ul.

Phenol extract (**FPT**).
Alcohol precipitate: 20 ul 3 M NaOAc, 400 ul EtOH (NO CARRIER).
After washing, be sure to dry pellet thoroughly.
Under direct observation, resuspend pellet in 3.5 ul 3X EE buffer by pipeting gently up and down. It should take several minutes to accomplish this. Avoid leaving droplets in pipet tip. The solution should be slightly viscous, and hold a tight meniscus. It may be cloudy, but should have no particulates. Final volume should be close to 4 ul.
Transfer to 0.2 ml PCR tube. Overlay with 1 drop oil.
Set one PCR block to 98° C, another to 67° C.
Preheat a small aliquot of 5 M NaCl at 67° C.
Incubate resuspended DNA at 98° C X 7 minutes.
Perform the following steps as quickly as possible:
Using Wheaton 0.2-2 ul "Socorex" micropipetor, preheat pipet tip in 5 M NaCl by pipeting up and down a few times.
Remove a 1ul aliquot of 5 M NaCl.
Remove DNA tube from 98° C, add the 1 ul of NaCl, pipet once or twice to mix, and place in 67° C block.
Incubate 40 hours at 67° C.

Hyb2 PCR1

Carefully remove as much oil as possible from the hybridization reaction.
Add 8 ul 5 mg/ml tRNA to Hyb2. Mix by pipetting up and down gently. Avoid getting droplets caught in pipet tip.
Add 10 ul loTE. Under direct observation, mix by pipeting gently until refraction lines are no longer seen.
Transfer to clean 1.5 ml microcentrifuge tube.
Wash hybridization tube with 80 ul loTE, and add to previous solution.
Add 300 ul loTE. Mix by pipeting gently (**FPT**). (400ul final volume)

100 uM NBgl 24	1.5 ul.....3.75
10X RDA	14.5 ul.....36.25
10mM dNTPs	6 ul.....15.0
50 mM $MgCl_2$	12 ul.....30.0
5 M Betaine	15 ul.....37.5
loTE	46 ul.....115

95 ul premix into each of two 0.2 ml PCR tubes.
50 ul diluted Hyb2 into each tube.
2 drops oil. Spin down.
Place in PCR machine and use "H2P1" program in SIM TUBE mode with a calibration factor of 200.
85° C X 2 minutes, hold at 72° C.
Add 5 ul 5 u/ul Taq. Mix thoroughly with second pipettor.
72° C X 5 minutes.
10 cycles: 94° C X 1 min, 72° C X 3 min.
Final incubation at 72° C X 5 min.

Mung Bean Nuclease Treatment of Hyb2-PCR1

Combine the two tubes of H2P1.
Phenol extract (**FPT**).
Alcohol precipitate (30 ul 3M NaOAc, 600 ul EtOH).
Resuspend pellet in 5 ul loTE (**FPT**).

Add:
- 4 ul 10X MBN buffer (NEB)
- 29 ul loTE
- 2 ul Mung Bean Nuclease (10 u/ul, NEB)

Mix gently with pipette.
Incubate 30 min at 30° C.
Spin down, then add 160 ul 50 mM Tris pH 8.8.
Phenol extract and alcohol precipitate.
Determine concentration by running an 2% agarose gel with 0.2, 0.4, 0.8 ug LMW markers and with 2ul, 5ul, 10ul 1:5 H2P1 amplicon dilution (2ul amplicon + 18uL loTE). Determine volume required for 50 ng.

Hyb2-PCR2

Test PCR:

10X RDA	14.5 ul.....50.75
10mM dNTPs	6 ul.....21
50 mM $MgCl_2$	12 ul.....42
5 M Betaine	15 ul.....52.5
loTE	66 ul.....231
100 uM NBgl24	1.5 ul.....5.25

Into 3 PCR tubes, add 115 uL premix and then add:

30 ul of digested amplicons to one tube
15 ul digested amplicons + 15 ul loTE to the next tube
to the last tube add 50 ng amplicons + loTE to bring final volume to 145 ul.
Add 2 drops oil, spin down.
Place in PCR machine and use "H2P2TST" program in SIM TUBE mode with a calibration factor of 200.
85° C X 3 minutes. Hold at 78° .
Add 5 ul 5 u/ul Taq in 1X RDA buffer.
Cycles: 94° C X 1 min, 72° C X 3 min.
12 cycles. Hold. Remove 15 ul.
3 more cycles (19). Hold. Remove 15 ul.
3 more cycles (22). Hold. Remove 15 ul.
3 more cycles (25). Hold. Remove 15 ul.
3 more cycles (28). Hold. Remove 15 ul.
3 more cycles (31).
Load 5 ul of aliquots on 2% gel. Choose the optimal number of cycles and DNA concentration to use.

Preparation of Round 2 Subtraction products

10X RDA	36.25 ul
10 mM dNTPs	15 ul
50 mM Mg Cl_2	30 ul
5 M Betaine	37.5 ul
100 uM NBgl24	3.75 ul
loTE	157.5 ul

115 ul premix each into 2 PCR tubes.
Add optimal diluted MBN-treated H1P1 + loTE to bring final volume to 145 ul.
2 drops oil
85° C X 3 min. Hold 78° C
Add 5 ul 5 u/ul Taq polymerase.
Use one less than the optimal number of cycles determined above.
After the cycles are over, prepare the following premix:

100 uM NBgl24	1.5 ul	→	3.75
10X RDA	1 ul		2.5
10 mM dNTP	2 ul		5
loTE	3.5 ul		8.75
5 u/ul Taq polymerase	2 ul		5

Preheat reaction tubes to 78° C.
Add 10 ul of premix to tubes.
Run 1 cycle: 94° C X 1 min, 70° C X 3 minutes, 72° C X 10 minutes.

Combine tubes.
Phenol extract (**FPT**).
Alcohol precipitate: 30 ul NaOAc, 600 ul EtOH. Do not add carrier.
Resuspend in 50 ul loTE (**FPT**). If the solution is cloudy, centrifuge 3 minutes and transfer the supernatant to a fresh tube.
Make a 1:5 dilution (4 ul into 16 ul loTE). Load 2,5 and 10 ul of 1:5 dilution of H1P2 with 5 ul of 5X loading buffer in a **3%** agarose gel with 0.2, 0.5 and 0.8 ug of LMW markers. Also load 1 ug of tester amplicons in adjacent lane. Determine DNA concentration of H2P2 stock. After gel electrophoresis, the repeat bands present in the tester amplicons should not be present in the H2P2 amplicons, but new additional distinct bands should be visible. If not, recheck amplicon concentrations and redo hybridization.

Digest 10 ug of H1P2 in 200 ul with 100 units Bgl II X 5 hours at 37°C, using buffer supplied by the manufacturer.
Phenol extract (**FPT**).
Concentrate amplicons on Centricon-100 device, and determine DNA concentration, as described in "Driver Amplicon Preparation".

Make two dilutions of stock digested amplicons: 20 ng/ul and 100 ng/ul.
At ligation step, prepare the following:
5 ul of digested amplicons (20 ng/ml or 100 ng/ml)
5 ul 100 uM JBgl24 (**purified**)
5 ul 100 uM JBgl 12 (**purified**)
3 ul 10X ligation buffer (NEB)
loTE to 29 ul final volume

Hybridize primers to DNA using 55° C hot block as described in "Genomic DNA Preparation" section.
Spin tubes down after 12° C, add 1 ul 400 u/ul T4 DNA ligase (NEB), and incubate at 30° C 8 hours to overnight.
Heat inactivate at 65° C for 10 minutes.
Add 50 ul 20 ug/ml tRNA to the ligation reactions.
Make an additional 1:5 dilution of the 100ng/ul ligation reaction (2 ul into 8 ul loTE).

Test PCR:

10X RDA	4.5 ul.....31.5
10mM dNTPs	2 ul.....14
50 mM $MgCl_2$	4 ul.....28
5 M Betaine	5 ul.....35
loTE	27.5 ul.....192.5

In 3 PCR tubes, place 0.5 ul 50 uM NBgl24 +0.5 uL loTE, in another 3 tubes place 0.5 ul 100 uM JBgl24 +0.5 uL loTE.
Add 43 ul of premix to each tube.

Add 1 ul of the 20 ng/ul ligation reaction to a tube with NBgl24 primer and to a tube with JBgl24 primer.
Add 1 ul of the 1:5 dilution of the 100 ng/ul ligation to a tube with JBgl24 primer and to a tube with NBgl24 primer.
Add 1 ul loTE to the remaining tubes (negative controls).
Add 2 drops oil to each tube. Spin.
Place in PCR machine and use "TEST72" program in SIM TUBE mode with a calibration factor of 100.
72° C X 3 min.
Add 5 ul 2 u/ul Taq in 1X RDA buffer.
72° C X 5 min.
15 cycles: 94° C X 1 min, 72° C X 3 min
Load 10 ul of each reaction on a 2% gel.
There should be an equal or greater amount of amplicons in the JBgl reaction than in the NBgl reaction. If not, redo tester amplicon preparation. Determine which ligation reaction yielded the best differential between J and N amplification, and/or the best yield of amplicons.

Hybridization #3

If the 20ng/ul ligation reaction is chosen, do the following dilutions:
8 ul of JBgl ligation (1.25 ng/ul) into 72 ul loTE (125 pg/ul)
2 ul of above dilution into 98 ul loTE (2.5 pg/ul)

If the 100ng/ul ligation reaction is chosen, do the following dilutions:
2 ul of JBgl ligation (6.25ng/ul) into 98 ul loTE (125 pg/ul)
2 ul of above dilution into 98 ul loTE (2.5 pg/ul)

In a clean 1.5 ml microcentrifuge tube, combine:
80 ug Bgl II digested driver amplicons
200 pg (80ul of 2.5 pg/ul dilution) of JBgl ligated round 2 subtraction amplicons
loTE to bring volume to 200 ul.

Phenol extract (**FPT**).
Alcohol precipitate: 20 ul 3 M NaOAc, 400 ul EtOH (NO CARRIER).
After washing, be sure to dry pellet thoroughly.
Under direct observation, resuspend pellet in 3.5 ul 3X EE buffer by pipeting gently up and down. It should take several minutes to accomplish this. Avoid leaving droplets in pipet tip. The solution should be slightly viscous, and hold a tight meniscus. It may be cloudy, but should have no particulates. Final volume should be close to 4 ul.
Transfer to 0.2 ml PCR tube. Overlay with 1 drop oil.
Set one PCR block to 98° C, another to 67° C.
Preheat a small aliquot of 5 M NaCl at 67° C.
Incubate resuspended DNA at 98° C X 7 minutes.
Perform the following steps as quickly as possible:

Using Wheaton 0.2-2 ul "Socorex" micropipetor, preheat pipet tip in 5 M NaCl by pipeting up and down a few times.
Remove a 1ul aliquot of 5 M NaCl.
Remove DNA tube from 98° C, add the 1 ul of NaCl, pipet once or twice to mix, and place in 67° C block.
Incubate 40 hours at 67° C.

Hyb3-PCR1

Carefully remove as much oil as possible from the hybridization reaction.
Add 8 ul 5 mg/ml tRNA to Hyb1. Mix by pipetting up and down gently. Avoid getting droplets caught in pipet tip.
Add 10 ul loTE. Under direct observation, mix by pipeting gently until refraction lines are no longer seen.
Transfer to clean 1.5 ml microcentrifuge tube.
Wash hybridization tube with 80 ul loTE, and add to previous solution.
Add 300 ul loTE. Mix by pipeting gently (**FPT**). (400ul final volume)

100 uM JBgl 24	1.5 ul.....3.75
10X RDA	14.5 ul.....36.25
10mM dNTPs	6 ul.....15.0
50 mM $MgCl_2$	12 ul.....30.0
5 M Betaine	15 ul.....37.5
loTE	46 ul.....115

95 ul premix into each of two 0.2 ml PCR tubes.
50 ul diluted Hyb3 into each tube.
2 drops oil. Spin down.
Place in PCR machine and use "H1P1" program in SIM TUBE mode with a calibration factor of 200.
85° C X 2 minutes (this step melts heteroduplexes that have hybridized to internal repetative elements).
Hold at 72° C.
Add 5 ul 5 u/ul Taq. Mix thoroughly with second pipettor.
72° C X 5 minutes (this step fills in the 3' end of hybridized DNA fragments)
10 cycles: 94° C X 1 min, 70° C X 3 min.
Final incubation at 72° C X 5 min.

Mung Bean Nuclease Treatment of Hyb3-PCR1

Combine the two tubes of H3P1.
Phenol extract (**FPT**).
Alcohol precipitate (30 ul 3M NaOAc, 600 ul EtOH).
Resuspend pellet in 5 ul loTE (**FPT**).

Add:
4 ul 10X MBN buffer (NEB)
29 ul loTE
2 ul Mung Bean Nuclease (10 u/ul, NEB)

Mix gently with pipette.
Incubate 30 min at 30° C.
Spin down, then add 160 ul 50 mM Tris pH 8.8.
Phenol extract and alcohol precipitate.
Determine concentration by running an 2% agarose gel with 0.2, 0.4, 0.8 ug LMW markers and with 2 ul, 5 ul, 10 ul 1:5 H3P1 amplicon dilution(2ul amplicon + 18uL loTE). Determine volume required for 50ng.

Hyb3-PCR2

Test PCR:

10X RDA	14.5 ul.....50.75
10mM dNTPs	6 ul.....21
50 mM $MgCl_2$	12 ul.....42
5 M Betaine	15 ul.....52.5
loTE	66 ul.....231
100 uM JBgl24	1.5 ul.....5.25

Into 3 PCR tubes, add 115 uL premix and then add:
30 ul of digested amplicons to one tube
15 ul digested amplicons + 15 ul loTE to the next tube
to the last tube add 50 ng amplicons + loTE to bring final volume to 145 ul
Add 2 drops oil, spin down.
Place in PCR machine and use "H1P2TST" program in SIM TUBE mode with a calibration factor of 200.
85° C X 3 minutes. Hold at 78° .
Add 5 ul 5 u/ul Taq in 1X RDA buffer.
Cycles: 94° C X 1 min, 70° C X 3 min.
16 cycles. Hold. Remove 15 ul.
3 more cycles (19). Hold. Remove 15 ul.
3 more cycles (22). Hold. Remove 15 ul.
3 more cycles (25). Hold. Remove 15 ul.
3 more cycles (28). Hold. Remove 15 ul.
3 more cycles (31).

Load 5 ul of aliquots on 2% gel. Choose the optimal number of cycles and DNA concentration to use.

Preparation of Round 3 Subtraction Products

10X RDA	72.5 ul
10 mM dNTPs	30 ul
50 mM $Mg Cl_2$	60 ul
5 M Betaine	75 ul
100 uM JBgl24	7.5 ul
loTE	315 ul

115 ul premix each into 4 PCR tubes.
Add optimal diluted MBN-treated H3P1 + loTE (if needed).
2 drops oil
85° C X 3 min. Hold 78° C
Add 5 ul 5 u/ul Taq polymerase.
Use one less than the optimal number of cycles determined above.
After the cycles are over, prepare the following premix:

100 uM JBgl24	1.5 ul	→	7.5
10X RDA	1 ul		5
10 mM dNTP	2 ul		10
loTE	3.5 ul		17.5
5 u/ul Taq polymerase	2 ul		10

Preheat reaction tubes to 78° C.
Add 10 ul of premix to tubes.
Run 1 cycle: 94° C X 1 min, 70° C X 3 minutes, 72° C X 10 minutes.

Combine tubes.
Phenol extract (**FPT**).
Alcohol precipitate: 30 ul NaOAc, 600 ul EtOH. Do not add carrier.
Resuspend in 50 ul loTE (**FPT**). If the solution is cloudy, centrifuge 3 minutes and transfer the supernatant to a fresh tube.
Make a 1:10 dilution (2 ul into 18 ul loTE). Load 2,5 and 10 ul of 1:5 dilution of H1P2 with 5 ul of 5X loading buffer in a **3%** agarose gel with 0.2, 0.5 and 0.8 ug of LMW markers. Also load 1 ug of tester amplicons in adjacent lane. Determine DNA concentration of H3P2 stock. After gel electrophoresis, the repeat bands present in the tester amplicons should not be present in the H1P2 amplicons, but new additional distinct bands should be visible. If not, recheck amplicon concentrations and redo hybridization.

For most applications, no more than 3 rounds of RDA are required. If an additional round is required, follow directions as for praparation of hybridization 1 subtraction products for hybridization 2, except use a much greater ratio of 1: 8 x 106 between tester and driver.

RDA amplicon preparation from microdissected samples

CAM 8/98

- Prepare 10 micron histologic frozen sections.
- Stain as per standard protocol for microdissection.
- Collect 5000 cells of tumor and benign cells in separate tubes.
- to each tube add 180 uL TE9 and 20 uL 10X TK buffer.
- Incubate overnight at 56°C.
- If using laser capture microdissection, cap tubes containing the buffers mentioned above using the capping tool. Invert tube and incubate in a 37° C incubator.
- Add 10 ul 10X TK buffer and continue incubation for a total of 24 hours or until no tissue particles remain.
- Cool tubes and spin down condensate.
- Add 4 uL 5 mg/mL tRNA.
- PCI extract.
- Alcohol precipitate: 20 uL # M NaOAc, 400 uL EtOH. 10 min. at - 20° C.10 minute spin.
- Wash pellets with 70% EtOH twice. Pipette off all liquid. Pellets can be briefly air-dried, but do not let pellets completely dry, or the genomic DNA may be difficult to resuspend.
- Resuspend DNA in 45 uL loTE and 5 uL of appropriate 10X restriction buffer. Add 20 units of desired restriction enzyme.
- 37°C X 2 hours.
- Spin down condensate and add 50 uL loTE and 2 uL 5 mg/mL tRNA.
- PCI extract.
- Alcohol precipitate: 10 uL 3 M NaOAc, 200 uL EtOH. 10 min. at - 20° C. 10 minute spin.
- Wash and dry as above.
- Resuspend in:
  10 uL 50 uM oligo24 (HPLC purified)
  10 uL 50 uM oligo12 (HPLC purified)
  6 uL loTE
  3 uL 10X ligation buffer
- Continue with regular RDA protocol.

RNA Extraction from LCM samples using Trizol (Life Technologies)

CAM 8/99

For most applications RNA will be extracted from cells obtained from frozen sectioning of tissue samples (see separate protocols for staining of frozen sections and for laser capture microdissection). Several thousand cells are extracted for most RNA applications, and several LCM caps may be used serially to pool the contents of multiple LCM sessions. RNA extraction is performed in the PCR room and hood.
All tubes and solutions used must be those designated for RNA use. All materials (including the outsides of stock bottles) must only be handled with gloves.

1. Place the LCM cap containing captured cells onto a 0.5 ml Eppendorf tube (with its cap cut off) which contains 400 µL Trizol (Life Technologies) and a small quantity of acid-washed 500 um glass beads (Sigma, G8772). Seat the cap using the LCM cap fitting tool. Invert and gently vortex the tube for 60 seconds to digest the tissue off of cap. Spin the tube briefly in a microcentrifuge to settle the contents. If combining several microdissection samples, remove the old LCM cap, replace with the next LCM cap, and repeat this procedure.
2. Transfer the contents to a 1.5 ml microfuge tube.
3. Add 80 ul chloroform (Note: do NOT add a chloroform:isoamyl alcohol mixture). Vortex to mix contents thoroughly, and let incubate at room temperature for 2 minutes.
4. Centrifuge for 15 min at 12,000 x g to separate the aqueous and organic phases.
5. Transfer upper aqueous layer to a new tube.
6. Add 100 ug of glycogen [20 ul of 5 mg/ml (Ambion)].
7. Add 200 µL isopropanol
8. Place 5 minutes on dry ice. Alternatively, the tube may be left at -20° C overnight.
9. Centrifuge for 30 min at 4° C at 12,000 x g, with caps hinges pointing outward so that the location of the pellet can be better predicted
10. Remove the majority of the supernatant with a 1000 µL tip and then switch to a smaller pipet to remove the rest of the supernatant. This minimizes disruption of the RNA pellet.
11. Wash with 0.5 ml 75% ethanol (4° C). Add the alcohol and spin for 3 min at 7000 x g at 4° C.
12. Remove the supernatant as explained above. All of the supernatant should be removed at this point.
13. Let the pellet air dry in the PCR hood to remove any residual ethanol. Do not over dry pellet.
14. Pellet can be stored in the -70° C freezer until further use.

DNase Treatment

1. To RNA pellet add (in order) 1 µL 40 U/µL RNase block (Stratagene), 15 µL RNase-free water, 2 µL 10X Exo III buffer (New England BioLabs). Resuspend pellet by pipeting well and gentle vortexing. Spin contents down.
2. Add 2 µL 10 U/µL DNase1 (RNase free, Stratagene).
3. Incubate at 37° C for 15 minutes.
4. Add1 ul Exonuclease III (New England BioLabs).
5. Incubate at 37° C for 15 minutes.
6. Add 30 ul RNase-free water.
7. Add 5 µL (0.1X volume) 2 M sodium acetate (pH 4.0).

8. Add 55 µL (1X volume) water saturated phenol (bottom layer) and 16.5 µL (0.3X volume) chloroform-isoamyl alcohol. Vortex vigorously.
9. Centrifuge 10 minutes.
10. Transfer upper layer to a new tube.
11. Add 50 ul isopropanol.
12. Continue as from step 9 above.